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Award Number: W81XWH-FF䔧䔧䔌ɪ €

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REPORT DATE: Œ*˘•ǿ́GFG

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
01-08-2012	Annual	1 Aug 2011 - 31 Jul 2012

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Patient-Specific B-Cell Antibody Factories to Treat Metastatic Disease	
	5b. GRANT NUMBER
	W81XWH-11-1-0440
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
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	5e. TASK NUMBER
E-Mail: claffey@nso2.uchc.edu	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
University of Connecticut Farmington, CT 06032	

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Breast cancer patients often require surgical resection of tumor along with the tumor-draining lymph nodes which are termed sentinel lymph nodes. These sentinel lymph nodes are evaluated for any tumor cells which have metastasized from the primary tumor nodule, indicating higher grade tumors and informing clinical treatments for systemic chemotherapy. In this contract, we will apply novel technologies to identify highly reactive immune cells in patient-derived sentinel lymph nodes that indicate a patient response to unknown tumor antigenic peptides. In this initial report we have confirmed methods to identify the antibody producing B-cells from patient lymph nodes, isolated them from the tissue and cultured them in the laboratory. In addition, we have tested the tools needed to now immortalize these cultured cells and select those that react to that patients own tumor proteins/peptides. Active enrollment and processing of patient samples was delayed due to administrative approval delays, both internal and external, clinical interface training and education to assure optimal sample viability and lack of the unique immortalization virus, Epstein Barr Virus (EBV). All of these initial hurdles have been overcome and we are actively engaged in the proposed contract activities as indicated in our original proposal.

15. SUBJECT TERMS
Breast Cancer, Sentinel lymph node, B-cell, EBV immortalization, Cancer Antigens

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			
U	U	U	UU	8	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction

Sentinel lymph nodes from breast cancer patients have B-cell antibody producing colonies which can be selected, cultured, expanded and immortalized to provide B-cell lines expressing anti-cancer antibodies selective for individual patients or breast cancer subtypes. This proposal is to develop the methodology and application to isolate these anti-cancer antibody producing B-cells and effectively immortalize them to produce antibody factories. Antibodies derived from these clones will be used to identify antigen distribution in breast cancers, breast cancer cell lines and other human malignancies. The ultimate goal is to obtain panel of antibodies to human breast cancer antigens designated as such by patient-specific immune biological responses as opposed to reverse genetic or proteomic methodologies currently being explored. The technological challenges within this proposal will be stepwise overcome with a focus on individual tasks provided in the Statement of Work. Current status of project was somewhat hampered by lack of commercial availability and internal instruction and education of clinical interface research office to increase patient enrollment over the period of the grant. Both issues currently resolved but induced a 4-5 month delay in optimal activity.

Revision of IRB protocol for collection under DOD award mechanism was delayed due to review by the Human Research Protection Office (HRPO) Office of Research Protections (ORP) U.S. Army Medical Research & Materiel Command (USAMRMC) Fort Detrick, Maryland. Currently we are now active to continue sample collection and processing.

Body

Task 1: Isolate, immortilize, select and expand B-cell antibody factories from 60 breast cancer patient sentinel lymph nodes. (Figures provided in below)

1a: Enrollment: Current enrollment during the period of the grant has been 6 samples after acquisition of reagents (backorder of EBV virus particles of 8 months) and research and clinical personnel training for collection of live samples which require alternative protocols to SOPs for operating room and pathology procedures. An average of 2 patients per month from Feb-April 2012.

1b: Analysis of sentinel lymph nodes for B-cell activation. Procedure for immunodetection of B-cell activation areas in a tissue print have been effective. Figure 1 demonstrates merged images of fluorescent signal for CD3 (T- and NK-cells) compared to CD23 (B-cell activation marker) on a tissue print of two nodes from a single case. Note that the circled areas are inversely correlated, that is high CD23 B-cell activation and low CD3 levels. Figure 2 shows distribution patterns in approximately 30% of two lymph nodes from the same patient. Although both nodes demonstrate many areas of follicles only 2-4 follicles are highly reactive as determined by CD23 expression. Figure 3 demonstrates even higher magnification immunofluorescence of specific signal for CD20 or CD23 combined with nuclear staining with DAPI to confirm cell density and pattern.

B-cell and non-B-cell core excision is performed on the live lymph node. An example of B-cell core excision is shown in Figure 4.

Cell culturing and analysis was performed with flow cytometry and cytospin-immunofluorescence. Determination of cell types in the initial cell pool collected using cytospin and immunofluorescence shows both CD3 T-cells and many CD20 B-cells. Some of the B-cells are positive for CD23 since this sample was from a CD23 positive core (Figure 5). However, a pan cytokeratin antibody showed no tumor cells. Additional cells identified by DAPI staining were

likely dendritic and reticular cells derived from the node sample. Figure 6 demonstrates the selectivity of the B-cell core over the non-B-cell core tissue cultured for 3 days demonstrating three-fold selectivity for B-cells.

1c: Immortilization using a anti-human IgG activated AKATA EBV cell supernatants was unsuccessful in expanding B-cells from this pilot experiment. No viable viral particles were found to be present in this sample and backordered commercial EBV has finally arrived (delayed 6 months from order!!!). Current stock already tested positive for EBV transmittance to human B-cell cell line.

Task 2: Identify and test antibody target expression in the source cancer, distribution in a large cohort of breast cancers and non-breast tumors using standard immunological methods.

2a-e: Immortilized antibodies from patient derived lymph nodes not available due to EBV particle unavailability. Currently on tract to capture and immortalize every sentinel lymph node case captured.

Task 3: Evaluate the effectiveness and selectivity of purified antibodies to target estrogen receptor alpha positive/negative breast tumors in vivo using real-time non-invasive imaging.

3a: A pilot analysis of fluorescent dye crosslinking to purified murine albumin has been performed and confirmed by gel electrophoresis (data not shown). Methods are in place to be incorporated with patient-derive antibodies.

Key Research Accomplishments

1) **Completion of all administrative, training, education and implementation of fresh sample collection approved by institution and the USAMRMC.**

2) **Detailed methods for throughput analysis of fresh samples are established as standard operating procedures.**

3) **Cell viability and primary culture conditions have been established to assure high specificity sampling, reactive B-cell content and viability in culture.**

4) **Tested current EBV reagent to assure effectiveness for transforming human B-cells.**

5) **Tested method to cross-link and purify antibodies with fluorescent dye needed to perform in vivo tumor targeting experiments.**

Reportable Outcomes

Manuscripts – none

Poster Presentations – UCONN Health Center Graduate Student Research Day "Identification of breast cancer sentinel lymph node activation", Charan Devorakonda PhD candidat

Conclusions

Despite a delay in effective throughput of patient samples due to unforeseen circumstances, we are on track to continue to pursue the aims and goals of the proposal.
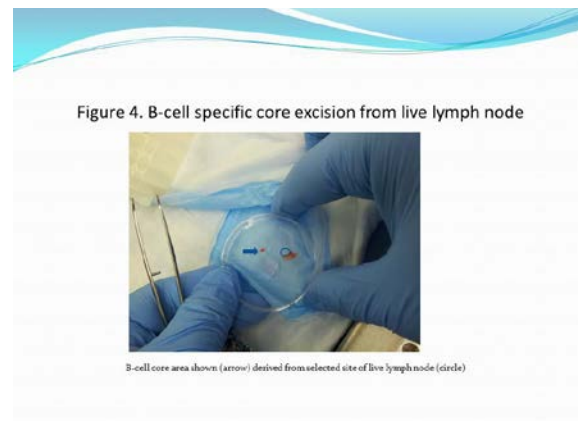
References

Figures

Figure 1. Tissue Print scans for areas of B-cell activation
As determined by CD23 and low CD3

Figure 2. Human Breast Cancer SLN Touch Preps:
B-cell (CD20), Active B-cell (CD23)

Figure 3. Representative highly activated B-cell germinal center targeted for collection

Localized active germinal center on tissue print of breast cancer sentinel lymph node

Figure 4. B-cell specific core excision from live lymph node

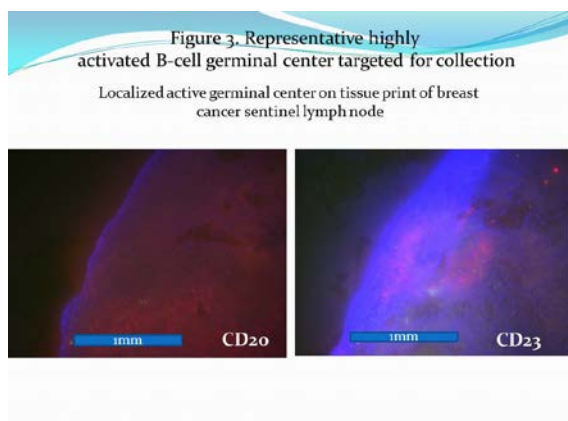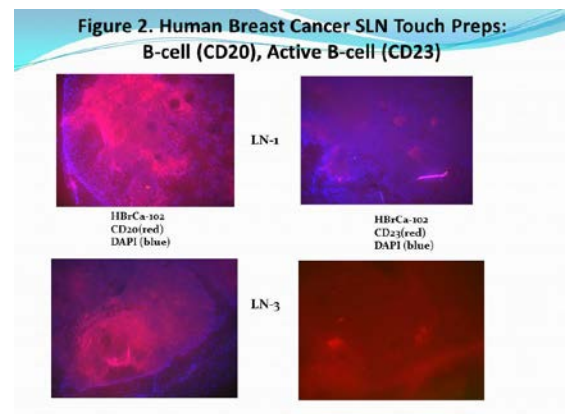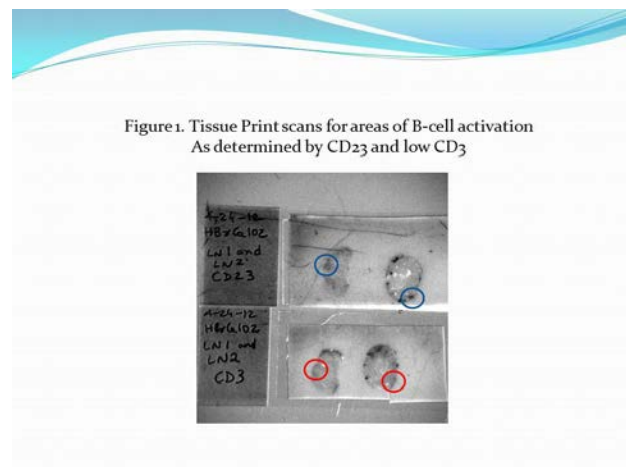B-cell core area shown (arrow) derived from selected site of live lymph node (circle)

Figure 5. Cytospin Analysis of B-cell Isolate in a Non-Metastatic Lymph Node
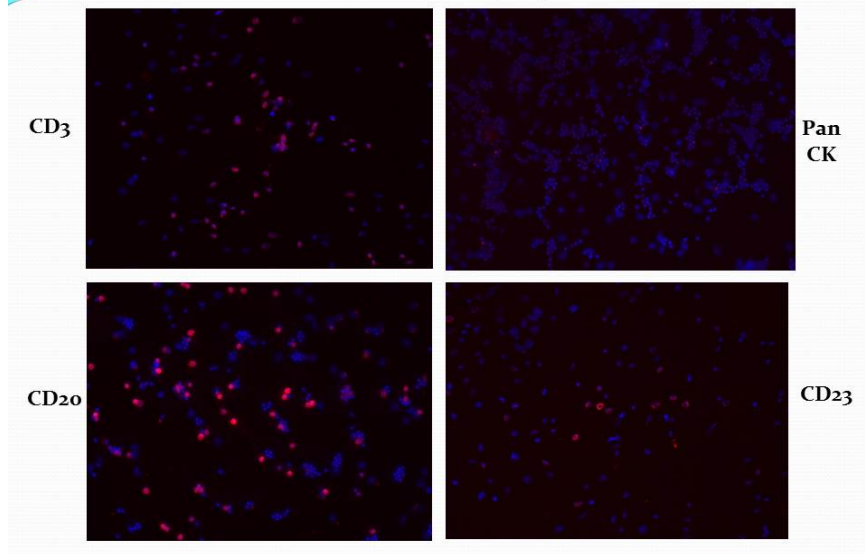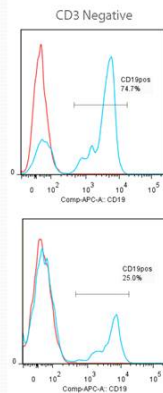

CD3

Pan CK

CD20

CD23

Figure 6. Flow cytometry analysis of the CD3 negative population in 3 day cultures of B-cell core or non-selective T-cell cores.

Appendices

Appendix: